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6-Methylhydroxylation of the anti-cancer agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) by flavin-containing monooxygenase 3

SHUFENG ZHOU[1], PHILIP KESTELL[2], JAMES W. PAXTON[1]

[1] Division of Pharmacology and Clinical Pharmacology, [2] Auckland Cancer Society Research Centre, The University of Auckland, Auckland, New Zealand

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SUMMARY

The involvement of flavin-containing monooxygenase (FMO) in the 6-methylhydroxylation of the experimental anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA) was investigated by use of human liver microsomes and microsomes containing cDNA-expressed FMOs. The involvement of FMO in the formation of 6-methyl hydroxylate of DMXAA, 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA) in human liver microsomes was indicated by the fact that this biotransformation was sensitive to heat treatment, increased at pH 8.3, and inhibited by methimazole. Only FMO3 formed 6-OH-MXAA at a similar rate to that in cDNA-expressed cytochromes P-450 (CYP)1A2. The results of this study indicate that human FMO3 has the capacity to form 6-OH-MXAA, but plays a lesser important role for this reaction than CYP1A2 that has been demonstrated to catalyse 6-OH-MXAA formation.

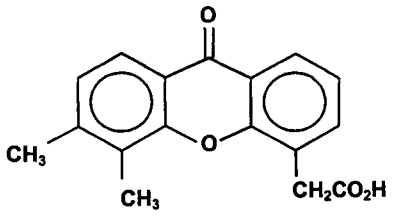
INTRODUCTION

5-6-Dimethylxanthenone-4-acetic acid (DMXAA) (Fig. 1), an anti-tumour drug with a novel mode of action, undergoes extensive hepatic metabolism in animals and humans (Webster et al., 1995; Miners et al., 1997; Kestell et al., 1999; Zhou et al., 2000), giving rise to two major metabolites, DMXAA acyl glucuronide (DMXAA-G), and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA), which are excreted into the bile and urine. The latter metabolic pathway in human liver microsomes has been reported to be catalysed by CYP1A2 (Zhou et al., 2000). However, further investigations have shown that 6-methylhydroxylation may also be catalyzed by flavin-containinng monoxygenase (FMO), as indicated by the inhibition of this reaction by methimazole (Zhou et al., 2000), an inhibitor of FMO.

FMOs are enzymes that catalyze NADPH-dependent oxygenation of nucleophilic nitrogen, sulfur, or phosphorus containing functional groups of xenobiotics (Hines et al.,

Please send reprint requests to: Dr Shufeng Zhou, PhD.
Division of Pharmacology and Clinical Pharmacology,
Faculty of Medical and Health Sciences,
The University of Auckland, Auckland, New Zealand

Abbreviations: CYP, cytochrome P-450; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; FMO, flavin-containing monooxygenase; HPLC, high performance liquid chromatography; 6-OH-MXAA, 6-hydroxymethyl-5-methylxanthenone-4-acetic acid.



5,6-dimethylxanthenone-4-acetic acid

Fig. 1: The chemical structure of DMXAA.

1994). Five distinct forms of FMO have been identified (FMO1 through FMO5) that are expressed in a tissue- and species-specific manner, with FMO3 the dominant isoform expressed in adult human liver microsomes (Cashman, 1995; Phillips et al., 1995). Human FMOs exhibit 82 to 88% sequence identity with their orthologs from other species, but only 51 to 57% sequence identity with each other (Phillips et al., 1995). FMOs often exhibit broad and overlapping substrate specificity, but substrate specificities exist among the different FMO families (Hines et al., 1994). The objective of this innvestigation was to examine the involvement of FMO in the metabolism of DMXAA.

Materials and Methods

Chemicals and reagents

DMXAA and the internal standard, 2,5-dimethylxanthenone-4-acetic acid (SN24350), were synthesised in the University of Auckland (Rewcastle et al., 1991). DMXAA was protected from light exposure to avoid degradation (Rewcastle et al., 1990). Authentic 6-OH-MXAA was isolated from rat urine, by previously published methods, and its structure identified by liquid chromatography mass spectrometry (LC-MS) and nuclear magnetic resonance spectrometry (Zhou et al., 2000). Bicinchoninic acid (BCA) reagent, NADPH and methimazole were purchased from Sigma-Aldrich Chemical Co. (Auckland, NZ). cDNA-expressed human microsomes containing FMO1, FMO3, and FMO5 were purchased from BD Gentest Co. (Woburn, MA). All othher reagents were of analytical or HPLC grade as appropriate.

Preparation of human liver microsomes

Human liver samples were obtained under strict ethical conditions from donors, and were stored at -80°C prior to use. Histological examination of the resected livers ensured the use of healthy liver tissue. Relevant details of the donors have been described elsewhere (Zhou et al., 2000). Ethical approval from the Northern New Zealand Research Ethics Committee and written informed consent for liver tissue to be used for research was obtained. Liver microsomes were prepared by differential centrifugation as described (Robson, 1987) and microsomes were stored at -80°C until used. Microsomal protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985). The CYP content was determined as described (Omura and Sato, 1964).

In vitro kinetic studies

The in vitro DMXAA 6-methylhydroxylation with human liver microsomes was performed using optimized incubation conditions (1mg/ml protein concentration and 40 min incubation time) (Zhou et al., 2000). Typical microsomal incubations (200 µl) for liver microsomal DMXAA 6-methylhydroxylation contained 1 mg/ml liver microsomal protein (0.5 mg/ml for cDNA-expressed microsomes), 0.5 mM NADPH, 5 mM $MgCl_2$, and DMXAA (5-350 µM) in 0.1 M phosphate buffer (pH 7.4), and were performed in triplicate. The reaction was initiated by the addition of NADPH and conducted at 37°C in a shaking water bath. After 40 min, incubations were stopped by cooling on ice and adding ice-cold 400 µl acetonitrile: methanol mixture (3:1, v/v) containing 2 µM internal standard (SN24350), and vortexing vigorously. Mixtures were centrifuged (3000 x g for 10 min) to remove the precipitated microsomal protein. The supernatant was removed and evaporated under nitrogen gas and the residue reconnstituted with 200 µl mobile phase. Fifty to seventy-five µl was injected into the HPLC for the measurement of 6-OH-MXAA. Within-day assay precision for the formation rate of 6-OH-MXAA for 5 separate incubations of the same batch of microsomes was less than 10% at DMXAA concentrations of 30 and 300 µM.

Incubation studies examining the effect of pH, enzyme Inhibitor, or heat on DMXAA 6-methylhydroxylation

Incubation conditions known to be optimal for FMO-mediated activity were utilized to examine the formation of 6-OH-MXAA by human liver microsomes. Microsomal incubations were performed with a mixture of human liver microsomes and 20 µM DMXAA at pH 7.4 and pH 8.3 respectively. Similar incubations with human liver microsomes (from HL12, HL13 and HL14) were performed at pH 7.4 in the presence or absence of methimazole (100 and 500 µM), an alternate-substrate competitive FMO inhibitor (Ring et al., 1999). To investigate the effect of heating at 55°C with or without

NADPH present, microsomes were heated at 55°C for 1 min, cooled on ice for approximately 2 min, and incubated with DMXAA.

HPLC and LC-MS

The method has been previously described (Zhou et al., 1999). Briefly, the HPLC system consisted of a solvent delivery system, a Model SF250 fluorescence detector (excitation and emission wavelengh, 345 nm and 409 nm, respectively), a Model 460 autosampler, and a Moder D450 data processing system (All from Kontron Instrument Co, Milan, Italy). A Luna C18 guard column and a 5 μm Spherex analytical column (150 x 4.6 mm; Phenomenex) were used. The mobile phase was the same as used for LC/MS except that the flow rate was 2.5 ml/min. The difference between the theoretical and measured concentrations, and the coefficient variation, were less than 15% at low quality control (QC) concentration (0.5 μM), and less than 10% at medium (2.5 μM) and high (10 μM) QC concentrations. Assay specificity was indicated by the absence of interfering chromatographic peaks in microsomal samples and in incubations with potential inhibitor. The LC-MS system was fitted with either an atmospheric pressure chemical ionisation or electrospray interface (Hewlett Packard, Avondale, PA, USA).

Data analysis

Data are presented as mean ± SD. Several models to describe the kinetics of DMXAA hydroxylation (single and two binding site, substrate-activator and substrate-inhibitor complex formation, and the sigmoid models) were fitted and compared using the Prism 3.0 program (Graphpad Software Co., CA, USA). The choice of model was confirmed by comparing and reviewing the relative residuals and the standard error of the parameter estimates from the non-linear regression analysis. The single binding

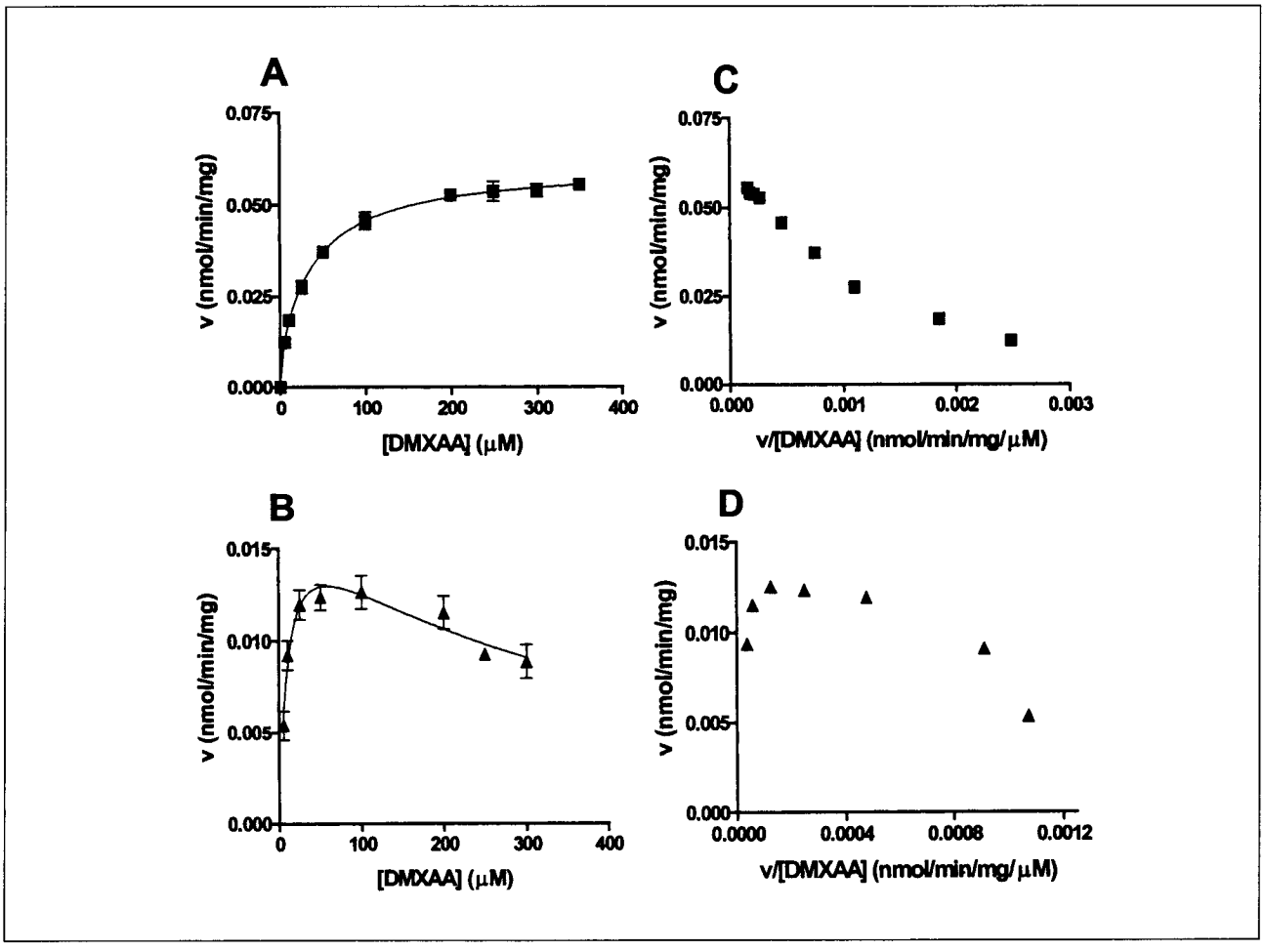


Fig. 2: Typical Michhaelis-Menten (A and B) and Eadie-Hofstee (C and D) plots for DMXAA 6-methylhydroxylation in human liver microsomes from HL12 (■) and cDNA-expressed FMO3 (▲). Each point represennts the mean ± SD of three determinations. The curves in A and B represent the fit of one enzyme binding-site model.

site model $v = V_{max} \cdot S/(K_m + S)$ gave the best fit, where v is the rate of hydroxylation; V_{max}, is the maximum velocity; K_m, the Michaelis-Menten constant; S, the substrate concentration. Correlation coefficients (r) were determined by linear regression with $p<0.05$ considered as statistically significant. The inhibitory constants (apparent K_i values) and the nature of inhibition were initially determined by Dixon plots, where K_i was given by the inntersection point of linear regression lines for data sets of 1/v against the concentration of inhibitor. The fitting of inhibition models (competitive, noncompetitive and uncompetitive) gave the final K_i value.

Results

6-Methylhydroxylation of DMXAA by cDNA-expressed FMO isoforms

Of the FMO isoenzymes investigated, only FMO3 exhibited catalytic activity toward the formation of 6-OH-MXAA. A comparison of the kinetics with native human liver microsomes is shown in Fig. 2 A & C (for Eadie-Hofstee plots, see Fig. 2B & D). A reduction in the rate of DMXAA 6-methylhydroxylation in cDNA-expressed FMO3 was observed at DMXAA concentration ≥100 μM. Substrate inhibition model was the best fit, with an apparent K_m of 9.7 ± 2.1 μM, V_{max} of 0.017 ± 0.001 nmol/min/mg, and K_{is} of 345 ± 80 μM. Under the HPLC conditions used, the major peak from the oxidation incubations had retention time identical to that of authentic 6-OH-MXAA. Further investigation using LC-MS showed that the molecular ions $[M+H]^+$ of this metabolite was m/z 299, which is consistent with the molecular weights of protonated 6-OH-MXAA. No activity was detected in microsomes containing FMO1, FMO5, or control microsomes containing the selectable plasmid vector without a cDNA insert.

Effect of pH, enzyme inhibitor, or heat on DMXAA 6-methylhydroxylation

The formation of 6-OH-MXAA was enhanced by 25% at pH 8.3 as compared with pH 7.4 in human liver microsomes. A decrease in 6-OH-MXAA formation to 57% of the control caused by preincubation at 55°C for 1 min in the absence of NADPH, suggests a heat-mediated inactivation of the FMO that was protected in the presence of NADPH, 6-OH-MXAA formation was inhibited in the presence of methimazole, with a K_i value of 61 μM (for Dixon plot, see Fig. 3).

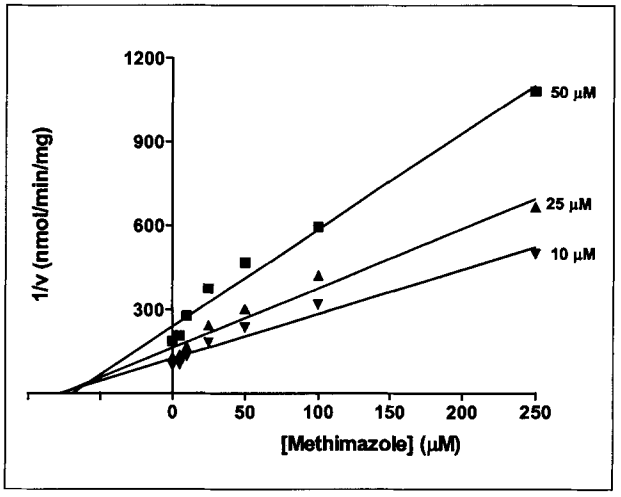


Fig. 3: Dixon plot for the inhibition of 6-methylhydroxylation in human liver microsomes by methimazole.

Discussion

This study indicates a role of FMO in the formation of 6-OH-MXAA inn human liver microsomes. This conclusion was reached through the examination of the formation of 6-OH-MXAA under a variety of incubation conditions. cDNA-expressed FMO3 formed this metabolite at a similar rate to that observed for expressed CYP1A2. As additional evidence of the role of FMO in this biotransformation byy liver microsomes, formation of 6-OH-MXAA exhibited characteristics of FMO-mediated reactions including sensitivity to heating treatment and enhanced activity at basic pH, and inhibition of the formation of 6-OH-MXAA by methimazole.

As in cDNA-expressed CYP1A2, the DMXAA 6-methylhydroxylation in cDNA-expressed FMO3 exhibited atypoical Michaelis-Menten kinetics with 2-3 fold lower apparent K_m and V_{max} values compared to those observed in native human liver microsomes, which may be due to differences in the phospholipid environment and lipid to protein ratio. Possible explanation for the reduction in 6-methylhydroxylation rate at DMXAA concentrations ≥ 100 μM in cDNA-expressed FMO3 include substrate inhibition, as indicated by the best fit of kinetics bt substrate inhibition model.

The involvement of both FMOs and CYPs is observed in the metabolism of a number of xenobiotics. However, the lack of inhibitor(s) selective for FMOs remains a major impediment to definitive investigations on the relative contributions of these enzymes in the metabolism of xenobiotics. The involvement of specific CYP isozymes in particular metabolic pathways is probed by using a wide array of in vitro techniques including selective chemical

inhibitors and specific antibodies. Comparable approaches for evaluating the role of FMOs include the determination of kinetics using the purified enzyme to complement well established characteristics of FMO-mediated reactions in microsomal preparations, including enzyme inhibition by methimazole, heat inactivation, and protection against heat inactivation by NADPH. The use of these techniques in the present study gave indication that FMO may play a minor role as methimazole (500 μM) inhibited only 35% of this reaction.

References

1. Cashman JR (1995) Structural and catalytic properties of the mammalian flavin-containing monooxygenase. Chem Res Toxicol 8: 165-181.
2. Hines RN, Cashman JR, Philpot RM, Williams DE and Ziegler DM (1994) The mammalian flavin-containing monooxygenases: Molecular characterization and regulation of expression. Toxicol Appl Pharmacol 125: 1-6.
3. Kestell P, Paxton JW, Rewcastle GW, Dunlop I and Baguley BC (1999) Plasma disposition, metabolism and excretion of the experimental antitumour agent 5,6-dimethylxanthenone-4-acetic acid in the mouse, rat and rabbit. Cancer Chemother Pharmacol 43: 323-330.
4. Miners JO, Valente L, Lillywhite KJ, Mackenzie PI, Burchell B, Baguley BC and Kestell P (1997) Preclinical prediction of factors influencing the elimination of 5,6-dimethylxanthenone-4acetic acid, a new anticancer drug. Cancer Res 57: 284-289.
5. Omura T and Sato R (1964) The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 23: 2370-2378.
6. Phillips IR, Dolphin CT, Clair P, Hadley MR, Hutt AJ, McCombie RR, Smith RL and Shephard EA (1995) The molecular biology of the flavin-containing monooxygenases of man. Chem-Biol Interact 96: 17-32.
7. Rewcastle GW, Atwell GI, Li ZA, Baguley BC and Denny WA (1991) Potential antitumour agents. 61. Structure-activity relationships for inn vivo colon 38 activity among disubstituted 9-oxo-9H-xanthenone-4-acetic acids. J Med Chem 34: 217-222.
8. Rewcastle GW, Kestell P, Baguley BC and Denny WA (1990) Light-induced breakdown of flavone acetic acid and xanthenone analogues in solution. J Natl Cancer Inst 82: 528-529.
9. Ring BJ, Wrighton SA, Aldridge SL, Hansen K, Haehner B and Shipley LA (1999) Flavin-containing monooxygenase-mediated N-oxidation of the M(1)-muscarinic agonist xanomeline. Drug Metab Dispos 27: 1099-1103.
10. Robson RA, Mathews AP, Miners JO, McManus ME, Meyer UA, Hall PD, Birkett DJ (1987) Characterisation of theophylline metabolism by human liver microsomes. Br J Clin Pharmacol 24: 293-300.
11. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Garter FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76-85.
12. Webster LK, Ellis AG, Kestell P and Rewcastle GW (1995) Metabolism and elimination of 5,6-dimethylxanthenone-4-acetic acid in the isolated perfused rat liver. Drug Metab Dispos 23: 363-368.
13. Zhou SF, Paxton JW, Tingle MD, McCall J and Kestell P (1999) Determinaton of two major metabolites of the novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid in hepatic microsomal incubations by high-performance liquid chromatography with fluorescennce detection. J Chromatogr (B) 734: 129-136.
14. Zhou SF, Paxton JW, Tingle MD and Kestell P (2000) Identification of the human liver cytochrome P450 isozyme responsible for the 6-methylhydroxylation of the novel anticancer drug 5,6-dimethylxanthenone-4-acetic acid. Drug Metab Dispos 28: 1449-1456.